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NICOTINAMIDE N-METHYLTRANSFERASE CATALYSES THE N-METHYLATION OF THE ENDOGENOUS β -CARBOLINE NORHARMAN: EVIDENCE FOR A NOVEL DETOXIFICATION PATHWAY

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ABSTRACT

Nicotinamide *N*-methyltransferase (NNMT) is responsible for the *N*-methylation of nicotinamide to 1-methylnicotinamide. Our recent studies have demonstrated that NNMT regulates cellular processes fundamental to the correct functioning and survival of the cell. It has been proposed that NNMT may possess β -carboline *N*-methyltransferase activity, endogenously and exogenously-produced pyridine-containing compounds which, when *N*-methylated, are potent inhibitors of Complex I and have been proposed to have a role in the pathogenesis of Parkinson's disease. We have investigated the ability of recombinant NNMT to *N*-methylate norharman to 2-*N*-methylnorharman. In addition, we have investigated the toxicity of the β -carboline norharman, its precursor 1,2,3,4-tetrahydronorharman and its *N*-methylated metabolite 2-*N*-methylnorharman, using our *in vitro* SH-SY5Y NNMT expression model. Recombinant NNMT demonstrated norharman 2*N*-methyltransferase activity, with a K_m of 90 ± 20 μ M, a k_{cat} of $3 \times 10^{-4} \pm 2 \times 10^{-5}$ s^{-1} and a specificity constant (k_{cat}/K_m) of 3 ± 1 $s^{-1} M^{-1}$. 1,2,3,4-Tetrahydronorharman was the least toxic of all three compounds investigated, whereas norharman demonstrated the greatest, with no difference observed in terms of cell viability and cell death between NNMT-expressing and non-expressing cells. In NNMT-expressing cells, 2-*N*-methylnorharman increased cell viability and cellular ATP concentration in a dose-dependent manner after 72 and 120 h incubation, an effect which was not observed after 24 h incubation or in non-NNMT-expressing cells at any time point. Taken together, these results suggest that NNMT may be a detoxification pathway for β -carbolines such as norharman.

SUMMARY STATEMENT

Nicotinamide *N*-methyltransferase has the ability to *N*-methylate the endogenously-produced β -carboline norharman, resulting in the production of 2-*N*-methylnorharman which demonstrated significantly lower toxicity *in vitro*.

SHORT TITLE

NNMT and norharman metabolism

KEYWORDS

β -Carbolines, biotransformation, methylation, detoxification, toxicology, enzyme kinetics

ABBREVIATIONS

BC, β -carboline; CxI, complex I; DAT, dopamine transporter; DMeNH, 2,9-dimethylnorharman; DV, dependent variable; FA, formic acid; hNNMT^{WT}, human wild-type nicotinamide *N*-methyltransferase; IV, independent variable; MeN, 1-methylnicotinamide; MeNH, 2-*N*-methylnorharman; MPP⁺, 1-methyl-4-phenylpyridinium ion; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NH, norharman; NNMT, nicotinamide *N*-methyltransferase; NNMT-V5, nicotinamide *N*-methyltransferase C-terminally fused with the V5 epitope; NPA, normalised peak area; PD, Parkinson's disease; PNMT, phenylethanolamine *N*-methyltransferase; PtPI, parent-to-product ion; THNH, 1,2,3,4-tetrahydronorharman; SAM, S-adenosylmethionine; SN, *substantia nigra*

INTRODUCTION

Nicotinamide *N*-methyltransferase (NNMT, E.C. 2.1.1.1) *N*-methylates nicotinamide to 1-methylnicotinamide (MeN) using *S*-adenosylmethionine (SAM) as cofactor [1]. It is widely expressed in human tissues, most strongly in the liver [2] and is upregulated in numerous cancers [3-6]. In the human brain, NNMT expression varies between regions and occurs solely in neurones, in particular within the dopaminergic neurones of the *substantia nigra* (SN) [2]. NNMT expression is also significantly elevated in the brains of patients who have died of Parkinson's disease (PD) [2,7]. Stable expression of recombinant NNMT in SH-SY5Y human neuroblastoma cells, which have no endogenous expression of NNMT, increases both Complex I (C_{xi}) activity and ATP synthesis [8], and promotes neurite branching and the formation of synapses *via* the activation of the Akt & ephrin B2 signalling pathways [9]. NNMT expression also protects against the toxicity of a variety of PD-relevant mitotoxins such as MPP⁺, rotenone and 6-hydroxydopamine [8,10]. These effects are replicated by MeN [8-10], suggesting that increased MeN production mediated the effects of NNMT.

A number of studies have proposed that NNMT has the ability to *N*-methylate both endogenous and exogenous compounds, for example tetrahydroisoquinolines and β -carboline (BCs), thereby generating inhibitors of C_{xi} [2,11,12]. BCs are found endogenously within the brain, where they arise from tryptamine metabolism [13,14], and as constituents/breakdown products in well-cooked foods such as meat and fish, roasted coffee, and tobacco smoke [15]. Due to the combination of their relatively high hydrophobicity and charge at physiological pH, BCs readily cross the blood-brain-barrier, whereupon they are *N*-methylated by as-yet unidentified *N*-methyltransferases [13,16,17]. They are considered to be possible mediators of SN degeneration because of their resemblance to 1-methyl-4-phenylpyridinium ion (MPP⁺) and their ability to inhibit C_{xi} [18-23]. Stereotaxic injection of BCs and their methylated derivatives into either the SN or the median forebrain bundle of rats resulted in striatal dopamine depletion, nigral lesions and gliosis [18,22,24] along with the onset of Parkinsonian-like motor symptoms [25]. In addition, the levels of BCs such as norharman (NH) have been shown to be elevated in the cerebral spinal fluid and SN of PD patients [24,26]. NH is a prototypical BC produced endogenously in the brain from 1,2,3,4-tetrahydronorharman (THNH) [27], which itself is produced *via* the demethylation of 1-methylTHNH [14,27,28]. The pyridine nitrogen of NH undergoes methylation to produce 2-*N*-methylnorharman (MeNH) [16] (Figure 1). MeNH is taken up selectively into dopaminergic neurones *via* the dopamine transporter (DAT) [23], where it weakly inhibits C_{xi} [11,18,29]. The toxicity of MeNH is increased by its subsequent *N*-methylation to 2,9-dimethylnorharman (DMeNH), the toxicity of which is reported to be comparable to that of MPP⁺ [20]. The concentration of DMeNH has been shown to be elevated in the cerebral spinal fluid of PD patients [21-23].

The identity of the enzymes responsible for these *N*-methylation reactions remains unknown. Gearhart *et al.* [30] demonstrated that phenylethanolamine *N*-methyltransferase (PNMT) has 2*N*-methyltransferase activity towards 9-*N*-methylnorharman, but 9-*N*-methylnorharman does not occur naturally within the brain. Therefore, the physiological relevance of this is unclear. No enzyme has been identified as being responsible for the initial 2-*N*-methylation of NH or for the subsequent 9-*N*-methylation to DMeNH. Several studies have suggested that NNMT may have NH 2*N*-methyltransferase activity and thus initiate the production of toxic *N*-methylated derivatives [2,7,31]. Although NNMT has been shown to be capable of *N*-methylating a variety

of non-physiological substrates [32], as yet there is no direct experimental evidence to demonstrate BC *N*-methyltransferase activity for NNMT. If NNMT does possess NH 2*N*-methyltransferase activity, it is possible that NNMT may increase the toxicity of NH, and as such may provide a mechanistic link between elevated NNMT expression and PD pathology.

To test this hypothesis, we assessed whether recombinant human NNMT possessed NH 2*N*-methyltransferase activity and compared the toxicity of THNH, NH and MeNH using our *in vitro* NNMT expression model.

EXPERIMENTAL PROCEDURES

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich (Poole, UK) and were of the highest grade available. Cell culture reagents were purchased from Life Technologies (Paisley, UK).

Synthesis of 2-*N*-methylnorharman

The synthesis of MeNH was based upon the method of Thatcher *et al.* [33]. Briefly, 500 mg NH were stirred with 10 mL acetonitrile (Fisher Scientific, Loughborough, UK) for 30 mins at RT, after which 0.5 mL methyl iodide were added and the reaction stirred at RT for 16 hours. The precipitate was filtered, washed with 5 mL diethyl ether three times, and dried under vacuum. A light yellow solid was obtained in a yield of 90%. A melting point of 236 – 238 °C was consistent with that previously reported [34]. ¹H-NMR spectrum (400 MHz (DMSO d₆)): δ 12.78 (1H, s, NH), 9.36 (1H, s, aromatic), 8.81 (1H, s, aromatic), 8.65 (1H, s, aromatic), 8.50 (1H, s, aromatic), 7.80 (2H, s, aromatic), 7.46 (1H, s, aromatic), 4.49 (3H, s, N⁺CH₃). ¹³C-NMR spectrum (100 MHz (DMSO d₆)): δ 143.9, 134.8, 134.8, 133.4, 131.87, 123.6, 121.5, 119.2, 117.6, 113.0, 47.7 (N⁺CH₃). Both NMR spectra matched those reported by Thatcher *et al.* [33]. *m/z* MeNH: 183.1 (C₁₂H₁₁N₂⁺).

Investigation of NNMT norharman 2*N*-methyltransferase activity

Cloning, expression and purification of recombinant human NNMT

Total RNA was isolated from normal human renal tissue using the SV Total RNA Isolation Kit (Promega, Madison, WI, USA) as per previously described [6]. Total RNA (2 µg) was reverse-transcribed into cDNA using M-MLV Reverse Transcriptase (Promega, Madison, WI, USA) using random primers. cDNA was subjected to PCR using the primers 5'-TCACATATGGAATCAGGCTTCA-3' (forward) and 5'-CTAAAGCTTTTCACAGGGGTCTG-3' (reverse) to amplify the human NNMT open reading frame which was cloned into a pET-28a plasmid vector to obtain the expression construct pET-28a-wt-hNNMT. This was transformed into *E. Coli* BL21 (DE3). Recombinant wild-type human NNMT (hNNMT^{WT}) was isolated from IPTG-induced cells and purified to homogeneity using nickel affinity purification as previously described [35]. Purity was confirmed using SDS-PAGE/Coomassie blue staining and NNMT identity was confirmed using SDS-PAGE/Western blotting as outlined in Table S1. Enzyme activity was 6740 nmoles 1-methylnicotinamide produced/h/mg protein.

Determination of MeNH and THNH parent-to-product ion transitions

The ability of NNMT to 2-*N*-methylate NH was assessed using a modification of our previously-described NNMT assay [36]. To take into account the possibility that the amount of MeNH produced was below the limit of detection for the assay, HPLC-UV detection of MeNH was

replaced with LC-MS/MS detection. To identify parent-to-product ion (PtPI) transitions for MeNH and THNH (internal standard), 1 µg/mL in 0.1% formic acid (FA)/50% methanol solutions of each compound were individually infused into a TSQ Quantum Access triple quadrupole mass spectrometer (Thermo Scientific, Loughborough, UK). Collision energies for MS-MS were 33 V and 12 V for NH and MeNH respectively. There were three PtPI transitions for MeNH (m/z): 183 → 168, 183 → 140 and 183 → 115, and a single PtPI transition for THNH: 173.1 → 144.

Enzyme assay

hNNMT^{WT} (21.25 ng/µL) was incubated with 1 mM NH, 50 mM Tris buffer (pH 8.6) and 1 mM dithiothreitol (all final concentrations) in a reaction volume of 760 µL for 5 mins at 37 °C using a pre-heated Eppendorf heater (Grant Instruments, Shepreth, UK), after which the reaction was initiated by the addition of 40 µL SAM (0.5 mM final concentration). Samples (150 µL) were taken immediately and subsequently every 15 minutes for 45 minutes, each of which were immediately added to 750 µL ice-cold acetonitrile to terminate the reaction. An aliquot (100 µL) of the terminated reaction solution was transferred to an extraction tube. THNH internal standard (166 ng/mL final concentration) was added, the solution was evaporated to dryness under N₂ at 40 °C for 30 mins and then reconstituted in 200 µL 0.1% FA/50% methanol solution. The reconstituted solution (10 µL) was injected into a Thermo Accela Pump and Autosampler (Thermo Scientific) coupled to a Thermo TSQ Quantum Access, and separated using a C4 Thermo Hypersil Gold 50x2.1 mm 1.9 µ column (Thermo Scientific) at a flow rate of 200 µL/min using the mobile phase gradient outlined in Table S2. Eluted reaction components were analysed using triple quadrupole positive electrospray ionisation, with a spray voltage of 3000 kV and a capillary temperature of 350 °C. MeNH and THNH were quantified using the peak areas of their respective 183 → 168 and 173.1 → 144 PtPI transitions, with confirmation of MeNH quantification using the 183 → 140 PtPI transition. MeNH peak area was normalised for THNH peak area to produce a normalised peak area (NPA) value, and MeNH production was calculated using a calibration curve constructed using MeNH standards of known NPA. MeNH production was calculated and expressed as nmol MeNH produced/mg protein. Linearity of reaction vs. time was determined by linear regression analysis using Prism v5.0 (GraphPad, San Diego, USA). Specific activity was calculated and expressed as nmoles MeNH produced/hour/mg protein ± SEM.

Kinetic analysis of NH 2N-methyltransferase activity

hNNMT^{WT} was incubated as described above with 0, 0.05, 0.1, 0.25, 0.5 and 1 mM NH. Samples were taken at 0, 30, 60, 120, 240 and 480 secs, from which the initial velocity was calculated and expressed as specific activity (nmoles MeNH produced /hour/mg hNNMT^{WT} protein). The kinetic constants K_m (µM ± SEM) and k_{cat} (s⁻¹ ± SEM) were calculated using non-linear Michaelis-Menten regression analysis, and were used to calculate k_{cat}/K_m which was expressed as s⁻¹ M⁻¹ ± SEM.

Characterisation of NNMT and PNMT expression

Cell culture

SH-SY5Y human neuroblastoma cells and S.NNMT.LP (SH-SY5Y cells stably expressing a recombinant NNMT C-terminally fused with the V5 epitope (NNMT-V5), produced as part of our ongoing studies) were cultured as previously described [8]. SH-SY5Y cells were chosen due

to their wide use in neurotoxicity studies [37] and their lack of endogenous NNMT expression [8]. SH-SY5Y of passage number 19 – 22 and S.NNMT.LP of passage number 7 – 9 (corresponding to SH-SY5Y passage number 22 – 24) were used for all experiments.

Western blotting and RT-PCR analysis of NNMT and PNMT expression

NNMT and PNMT mRNA expression in SH-SY5Y and S.NNMT.LP was assessed using RT-PCR as previously described [8] using primers outlined in Table S3. Recombinant NNMT-V5 and endogenous PNMT protein expression was detected using SDS-PAGE/Western blotting as previously described [8] using combinations of primary and secondary antibodies outlined in Table S1. As a positive control for PNMT expression, Balb/c mouse whole brain homogenate, obtained from the Biomedical Services Unit, KCL, was prepared from mice euthanized as per Schedule 1 of the Animal (Scientific Procedures) Act (1986) as previously described [9].

Investigation of THNH, NH and MeNH toxicity

MTT reduction assay

Cells were seeded into the wells of a 96-well plate in quadruplicate (Thermo Scientific, Loughborough, UK) at a density of 15,000 cells/well and incubated with serial dilutions of test compounds (THNH: 1600 – 12.5 μ M; NH: 800 – 6.3 μ M; MeNH: 2000 – 15.6 μ M); these concentration ranges were optimised to ensure adequate statistics in the subsequent toxicity dose-response data analysis. After 24, 72 and 120 h incubation, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction was assessed as previously described [38] and results were calculated and expressed as percentage cell viability compared to untreated cells \pm SEM ($n = 5$ for THNH and NH, $n = 4$ for MeNH). To calculate EC50 for each compound, concentration was log-transformed, and analysed using non-linear regression analysis using the following equation:

$$y = \text{minimum} + \frac{\text{maximum} - \text{minimum}}{1 + 10^{(\text{LogEC50} - x) * \text{hillslope}}}$$

and a constraint of $y = 0$ for the minimum.

LDH cytotoxicity assay

Cells were seeded and incubated with test compounds as described above for 120 h, after which lactate dehydrogenase (LDH) release was measured using the Pierce LDH Cytotoxicity Assay kit (Thermo Fisher Scientific, Paisley, UK) as per manufacturer's instructions. LDH release was calculated and expressed as percentage cell death \pm SEM ($n = 3$). Data analysis and calculation of LC50s was performed using non-linear regression as described above, using a constraint of $y = 100$ for the maximum.

Cellular ATP content assay

Cells were seeded into the wells of a poly-L-lysine-pretreated white 96-well plate (Greiner Bio-One Ltd., Stonehouse, UK) at a density of 15,000 cells/well in triplicate. Cells were incubated with test compounds as described above for 120 h, after which ATP content was measured as previously described [8] and expressed as percentage ATP content compared to untreated cells \pm SEM ($n = 5$ for THNH and NH, $n = 4$ for MeNH). Data analysis and calculation of EC50s was performed as described for the MTT assay.

Statistical analysis

All statistical analysis was undertaken using Prism (Graphpad, San Diego, CA, USA). In all instances, significance was taken at $P < 0.05$. To determine the overall effect of each compound dose upon MTT reduction, cellular ATP content and LDH release compared to untreated cells for each cell line, a 1-way repeated measures ANOVA with Tukey *post hoc* comparisons using blocked, raw mean data for each experiment was performed with concentration as the independent variable (IV) and absorbance minus background (MTT and LDH) or luminescence minus background (ATP) as the dependent variable (DV) [39]. To determine cell line differences in toxicity for individual concentrations, a 2-way repeated measures ANOVA using blocked, mean data for each experiment with concentration and cell line as IV and data normalised as percentage of untreated cells as DV. Normalisation of the DV takes into account differences in baseline MTT reduction, cell death and cellular ATP differences of untreated cells between both cell lines [8,10]. As such, untreated cells were not included in this analysis as they would be expressed as $100 \pm 0\%$. Unless otherwise stated, all P values are <0.001 . To determine differences in compound toxicity, logEC50 or logLC50 was determined for each experimental repeat which were subsequently averaged to obtain logEC50s \pm SEM and logLC50s \pm SEM and have been summarised in Table S4. These were then compared using Student's *t*-test (comparison in both cell lines) or 1-way ANOVA with Tukey *post hoc* comparisons (comparison of all three compounds or time points in a single cell line). The use of logEC50s and logLC50s, which follow a normal distribution, mitigates against the asymmetry that exists when the antilog of a log standard error is obtained [40].

RESULTS

NNMT demonstrated norharman 2N-methyltransferase activity

To determine whether NNMT has BC N-methyltransferase activity, we investigated whether recombinant hNNMT^{WT} could N-methylate NH (Figure 2). Incubation of hNNMT^{WT} with 1 mM NH resulted in the production of MeNH, which increased linearly with time (Figure 2a), providing a calculated specific activity of 37 ± 2 nmoles MeNH produced/hr/mg protein. Estimation of the kinetic parameters K_m and k_{cat} using Michaelis-Menten non-linear regression of enzyme incubated with varying concentrations of NH (Figure 2b) provided an estimated K_m of 90 ± 20 μ M and a K_{cat} of $3 \times 10^{-4} \pm 2 \times 10^{-5}$ s⁻¹ respectively. k_{cat}/K_m was calculated to be 3 ± 1 s⁻¹ M⁻¹.

PNMT protein expression was undetectable in both SH-SY5Y and S.NNMT.LP cells

As previously described [8], neither endogenously expressed NNMT mRNA nor recombinant NNMT-V5 were detected in SH-SY5Y cells, but recombinant NNMT-V5 mRNA and protein, however, were expressed robustly in S.NNMT.LP cells (data not shown). PNMT mRNA was expressed in both cell lines (Figure 3a), but PNMT protein was undetectable in either cell line (Figure 3b).

BCs demonstrated differential toxicity towards SH-SY5Y and S.NNMT.LP cells

Cell viability – MTT assay

The toxicity of THNH, NH and MeNH was compared in SH-SY5Y and S.NNMT.LP after 120 h incubation using the MTT cell viability assay (Figure 4 and Tables 1 & 2), an early stage measure of toxicity [42]. THNH was significantly toxic towards SH-SY5Y cells at 400 ($P <$

0.05), 800 and 1600 μM , and towards S.NNMT.LP at the same concentrations. No significant difference in toxicity was observed between cell lines at any individual dose. There was no significant difference in $\text{EC}_{50_{\text{THNH}}}$ for each cell line ($P = 0.19$). NH was toxic towards SH-SY5Y cells at 100 ($P < 0.05$), 200, 400 and 800 μM , and towards S.NNMT.LP at 200, 400 and 800 μM . No significant difference in toxicity was seen between cell lines at any individual concentration. There was no significant difference in $\text{EC}_{50_{\text{NH}}}$ for each cell line ($P = 0.78$). MeNH was toxic towards SH-SY5Y cells at 250, 500, 1000 and 2000 μM . MeNH demonstrated toxicity towards S.NNMT.LP at 500, 1000 and 2000 μM , and increased cell viability at 62.5 and 125 μM . There was a significant difference in toxicity towards SH-SY5Y and S.NNMT.LP at 125 μM ($93 \pm 3\%$ vs. $164 \pm 7\%$) and 250 μM ($55 \pm 2\%$ vs. $92 \pm 6\%$, $P < 0.01$). The $\text{EC}_{50_{\text{MeNH}}}$ was significantly lower in SH-SY5Y than S.NNMT.LP ($P = 0.009$).

For SH-SY5Y cells (Figure S1), there were significant differences in the EC_{50} s for all compounds, with $\text{EC}_{50_{\text{THNH}}}$ greater than both $\text{EC}_{50_{\text{NH}}}$ and $\text{EC}_{50_{\text{MeNH}}}$. Likewise, $\text{EC}_{50_{\text{MeNH}}}$ was significantly greater than $\text{EC}_{50_{\text{NH}}}$. For S.NNMT.LP cells (Figure S1), $\text{EC}_{50_{\text{THNH}}}$ was significantly greater than $\text{EC}_{50_{\text{NH}}}$, and $\text{EC}_{50_{\text{MeNH}}}$ was greater than $\text{EC}_{50_{\text{NH}}}$. In contrast, $\text{EC}_{50_{\text{THNH}}}$ was not significantly different to $\text{EC}_{50_{\text{MeNH}}}$.

Cell death – LDH release

We next investigated the effect of BCs upon cell death after 120 h incubation as measured by the LDH assay (Figure 5 and Tables 1 & 2), an end stage measure of toxicity [41]. THNH was toxic towards both SH-SY5Y and S.NNMT.LP cells at 1600 μM only. THNH was significantly more toxic towards SH-SY5Y than S.NNMT.LP cells at 400 μM ($27 \pm 3\%$ vs. $13 \pm 0.6\%$) and 1600 μM ($58 \pm 10\%$ vs. $34 \pm 2\%$). There was no significant difference in $\text{LC}_{50_{\text{THNH}}}$ for each cell line ($P = 0.12$). NH was toxic towards both SH-SY5Y and S.NNMT.LP cells at 200, 400 and 800 μM . Cell death was significantly higher in SH-SY5Y compared to S.NNMT.LP cells at 400 μM ($85 \pm 6\%$ vs. $54 \pm 0.6\%$). The $\text{LC}_{50_{\text{NH}}}$ was significantly lower in SH-SY5Y compared to S.NNMT.LP ($P = 0.0006$). MeNH was toxic towards SH-SY5Y cells at 2000 μM only, whereas MeNH was toxic towards S.NNMT.LP cells at 500, 1000 and 2000 μM . Cell death was significantly lower in SH-SY5Y compared to S.NNMT.LP cells at 500 μM ($34 \pm 2\%$ vs. $99 \pm 3\%$), 1000 μM ($31 \pm 6\%$ vs. $84 \pm 4\%$) and 2000 μM ($73 \pm 2\%$ vs. $89 \pm 5\%$). The $\text{LC}_{50_{\text{MeNH}}}$ was significantly higher in SH-SY5Y compared to S.NNMT.LP cells ($P = 0.0002$).

For SH-SY5Y (Figure S2), the $\text{LC}_{50_{\text{NH}}}$ was significantly lower than both the $\text{LC}_{50_{\text{THNH}}}$ and $\text{LC}_{50_{\text{MeNH}}}$, with no significant difference between $\text{LC}_{50_{\text{THNH}}}$ and $\text{LC}_{50_{\text{MeNH}}}$. In contrast, for S.NNMT.LP cells (Figure S2) $\text{LC}_{50_{\text{THNH}}}$ was significantly greater than both $\text{LC}_{50_{\text{NH}}}$ and $\text{LC}_{50_{\text{MeNH}}}$. There was no significant difference between $\text{LC}_{50_{\text{NH}}}$ and $\text{LC}_{50_{\text{MeNH}}}$.

Cellular ATP content assay

The effect of BCs upon cellular ATP content after 120 h incubation was assessed (Figure 6 and Tables 1 & 2). THNH reduced cellular ATP content in SH-SY5Y cells at 400, 800 and 1600 μM . Likewise, THNH reduced cellular ATP content in S.NNMT.LP cells at the same concentrations. No significant difference in cellular ATP content was observed between cell lines at any individual dose. There was no significant difference in $\text{EC}_{50_{\text{THNH}}}$ for each cell line ($P = 0.21$). NH reduced cellular ATP content in SH-SY5Y cells at 400 and 800 μM , whereas in S.NNMT.LP NH reduced cellular ATP content at 200, 400 and 800 μM . There was a significant

difference in cellular ATP content in SH-SY5Y and S.NNMT.LP at 200 μ M ($86 \pm 15\%$ vs. $39 \pm 5\%$). The $EC_{50_{NH}}$ was significantly higher in SH-SY5Y compared with S.NNMT.LP cells ($P = 0.02$). MeNH reduced cellular ATP content in SH-SY5Y cells at 250, 500, 1000 and 2000 μ M. In S.NNMT.LP, MeNH reduced cellular ATP content at 500, 1000 and 2000 μ M, and increased cellular ATP content at 125 μ M ($P < 0.05$). Cellular ATP content was significantly lower in SH-SY5Y than S.NNMT.LP at 125 μ M ($74 \pm 9\%$ vs. $140 \pm 14\%$) and 250 μ M ($46 \pm 16\%$ vs. $110 \pm 11\%$). The $EC_{50_{MeNH}}$ was significantly lower in SH-SY5Y compared with S.NNMT.LP cells ($P = 0.009$).

For SH-SY5Y (Figure S3), the $EC_{50_{THNH}}$ was significantly greater than both $EC_{50_{NH}}$ and $EC_{50_{MeNH}}$, with no difference between $EC_{50_{NH}}$ and $EC_{50_{MeNH}}$. For S.NNMT.LP (Figure S3), the $EC_{50_{THNH}}$ was greater than $EC_{50_{NH}}$, as was $EC_{50_{MeNH}}$ compared to $EC_{50_{NH}}$. In contrast, the $EC_{50_{THNH}}$ was not significantly different to $EC_{50_{MeNH}}$.

Effect of length of exposure upon cell viability

Finally, we investigated whether BCs demonstrated time-dependent differences in cell viability as measured using the MTT reduction assay (Figure 7 and Tables 1 & 2). THNH was toxic towards SH-SY5Y cells after 24 h incubation at 400, 800 and 1600 μ M. Likewise, after 72 h incubation THNH was toxic at the same concentrations. THNH was toxic towards S.NNMT.LP cells after 24 h incubation at concentrations of 200 μ M and greater. After 72 h incubation, THNH was significantly toxic at concentrations of 400, 800 and 1600 μ M. THNH did not increase MTT reduction at any concentration or time point in either cell line. There was no significant difference in $EC_{50_{THNH}}$ towards both SH-SY5Y and S.NNMT.LP cells over the time course of the experiment. There were no significant differences in $EC_{50_{THNH}}$ at each time point between SH-SY5Y and S.NNMT.LP cells.

NH was toxic towards SH-SY5Y cells after 24 h incubation at 100 ($P < 0.01$), 200, 400 and 800 μ M. After 72 h incubation, NH was toxic at the same concentrations. Likewise, NH was toxic towards S.NNMT.LP cells after 24 h incubation at concentrations of 100 μ M and greater. After 72 h incubation, NH was toxic at 200 ($P < 0.01$), 400 and 800 μ M. NH did not increase MTT reduction at any concentration or time point in either cell line. There were no significant differences in $EC_{50_{NH}}$ towards both cell lines over the time course of the experiment. There was no significant difference in $EC_{50_{NH}}$ at each time point between SH-SY5Y and S.NNMT.LP.

MeNH was toxic towards SH-SY5Y cells after 24 h incubation at 500 ($P < 0.01$), 1000 and 2000 μ M. After 72 h incubation, MeNH was toxic at 1000 and 2000 μ M. MeNH was toxic towards S.NNMT.LP cells after 24 h incubation at 500, 1000 and 2000 μ M. After 72 h incubation, MeNH was toxic at 1000 and 2000 μ M. MeNH did not increase MTT reduction in SH-SY5Y at any of the concentrations or time points investigated, however MTT reduction was increased in S.NNMT.LP after 72 h incubation with 31.3 and 62.5 μ M ($P < 0.05$) MeNH. There were significant differences in $EC_{50_{MeNH}}$ over the time course of the experiment. In SH-SY5Y cells, $EC_{50_{MeNH}}$ decreased with increasing length of incubation. In S.NNMT.LP cells, $EC_{50_{MeNH}}$ was not significantly different after 24 and 72 h incubation, however $EC_{50_{MeNH}}$ was significantly lower after 120 h incubation ($P < 0.05$). After 24 h incubation, MeNH was significantly less toxic towards SH-SY5Y than S.NNMT cells ($P = 0.006$), however due to the increasing toxicity of MeNH towards SH-SY5Y with increasing length of incubation, there was no significant

difference in $EC_{50_{MeNH}}$ observed between SH-SY5Y and S.NNMT.LP cells after 72 h incubation and was significantly more toxic towards SH-SY5Y than S.NNMT.LP after 120 h incubation ($P = 0.008$).

In SH-SY5Y cells after 24 h incubation, $EC_{50_{MeNH}}$ was greater than both $EC_{50_{THNH}}$ ($P < 0.01$) and $EC_{50_{NH}}$, with $EC_{50_{THNH}}$ greater than $EC_{50_{NH}}$ ($P < 0.01$). Likewise, after 72 h incubation, $EC_{50_{MeNH}}$ was greater than both $EC_{50_{THNH}}$ ($P < 0.05$) and $EC_{50_{NH}}$ ($P < 0.01$), with $EC_{50_{THNH}}$ greater than $EC_{50_{NH}}$ ($P < 0.05$). In S.NNMT.LP cells after 24 h incubation, there was no significant difference between $EC_{50_{MeNH}}$ and $EC_{50_{THNH}}$, whereas both $EC_{50_{THNH}}$ and $EC_{50_{MeNH}}$ were significantly greater than $EC_{50_{NH}}$ ($P < 0.01$ for both). After 72 h incubation, there was no significant difference between $EC_{50_{THNH}}$ and $EC_{50_{NH}}$, whereas both $EC_{50_{THNH}}$ and $EC_{50_{NH}}$ were lower than $EC_{50_{MeNH}}$.

DISCUSSION

The potential for NNMT to *N*-methylate endogenously and exogenously-derived compounds such as BCs into inhibitors of CxI been proposed previously [2,7,12] but has not been demonstrated hitherto. This study is the first to demonstrate that (1) NNMT has NH 2*N*-methyltransferase activity and (2) MeNH is less toxic than NH.

NNMT demonstrated norharman 2*N*-methyltransferase activity

The demonstration of NH 2*N*-methyltransferase activity for NNMT (Figure 2) is the first direct experimental evidence of such towards an endogenously-produced substrate other than nicotinamide, and is a further addition to the list of pyridine-containing compounds which are substrates for NNMT [32]. Comparison of the kinetic parameters for NNMT reveals that NNMT has a lower K_m yet lower k_{cat} and k_{cat}/K_m towards NH compared to nicotinamide. Our own studies of human NNMT, using whole liver homogenate as source, reported a K_m of 1.36 mM for nicotinamide, 15-times higher than that for NH reported here [36]. Studies by Rini *et al.* [42] of human liver NNMT demonstrated a similar trend, with a K_m of 350 μ M (4-times higher). We have previously reported a K_m of 380 – 430 μ M for the hNNMT^{WT} used in this study [35], 4.4 – 4.9-fold higher than the K_m for NH. The k_{cat} for NH is 265-fold lower than that for nicotinamide [35]. Such a low k_{cat} is not without precedence. The DNA endonuclease I-Crel and γ -secretase have very low catalytic turnover towards their endogenous substrates, as evidenced by k_{cat} values of $4 \times 10^{-4} \text{ s}^{-1}$ and $1.2 \times 10^{-3} \text{ s}^{-1}$ respectively [43,44]. Furthermore, the amino acid transaminase activity of tyrosine phenol-lyase is similar to that which we report for the *N*-methylation of NH, with k_{cat} in the range $1 \times 10^{-4} - 2 \times 10^{-3} \text{ s}^{-1}$ dependent upon substrate [45]. Such a low k_{cat} indicates that the K_m for the *N*-methylation of NH is close to k_s , the equilibrium dissociation constant for the NNMT-NH complex, suggesting that NH is not a good substrate for NNMT. This is supported by comparing k_{cat}/K_m of NNMT for NH with that of nicotinamide, which is 54-fold larger [35]. Thus, it is clear that nicotinamide is the preferred substrate for the enzyme and, within the cell, NH metabolism may be inhibited by nicotinamide *via* competitive inhibition. The rate at which BCs are endogenously produced is unknown, although it is likely that both BC and nicotinamide levels will fluctuate due to dietary intake [46]. Hence, it is not possible to state with certainty that the intracellular concentrations of nicotinamide will be in excess of NH at all times; therefore, whilst NNMT can *N*-methylate NH, the degree to which this occurs in the cell is uncertain.

In contrast to what we have previously observed for nicotinamide, NNMT does not appear to demonstrate substrate inhibition kinetics when incubated with NH (Figure 2B) [36]. This may arise due to the differences in NNMT preparation used in the respective studies, i.e. whole liver homogenate *vs.* purified recombinant protein. Also, it is possible that incubation with higher concentrations of NH may yet reveal substrate inhibition. Nonetheless, these results do suggest differing regulation of NNMT's various enzyme kinetic activities which may reflect their respective metabolic requirements.

The expression of NNMT-V5 influenced the toxicity of BCs

No study has investigated the effect of the expression of an enzyme known to possess BC *N*-methyltransferase activity upon their toxicities. In our present study, THNH – the precursor of NH – was the least toxic of all three compounds investigated, and its overall toxicity (as measured using EC50s and LC50) was not influenced by the expression of NNMT-V5 (Figures 4 – 7). Tetrahydro-BC alkaloids such as THNH and 1-methyl-THNH carboxylate occur in foods such as wine, seasonings and fruit products, and possess radical scavenging properties [47]. Injection of 2-*N*-methyl-THNH, the aliphatic analogue of MeNH, into rat brain has no effect on dopaminergic neurone viability or function [48], which taken together with our results demonstrate that the pyridinium moiety is essential for BC toxicity.

In contrast, NNMT-V5 expression influenced the toxicity of NH. The EC50_{ATP} for NH was lower in S.NNMT.LP compared to that of SH-SY5Y cells (Figure 5), possibly due to the increased *N*-methylation of NH in S.NNMT.LP cells by NNMT-V5. This *N*-methylation most likely occurs *via* NNMT-V5 as evidenced by the lack of PNMT expression in either cell line. Since MeNH is a weak CxI inhibitor, although with a 7-fold higher IC50 than MPP+, it is also possible that the increased toxicity of NH (in terms of reduction in cellular ATP concentration) in S.NNMT.LP cells may arise due to its inhibition of Complex III [11]. Conceivably, this inhibitory effect would be greater in S.NNMT.LP cells as the rate of oxidative phosphorylation is increased compared to that in SH-SY5Y cells [8]. NH toxicity, however, is thought to be mediated predominantly *via* the formation of DMeNH [22], making the inhibition of Complex III by NH unlikely to be responsible for the effect observed.

Of significance was the observation that the EC50_{NH} for cell viability (as measured using the MTT assay, Figure 7) towards each cell line after 120 h incubation was not significantly different, whereas its LC50 after 120 h incubation (Figure 5) was higher in S.NNMT.LP cells, demonstrating that the decrease in cellular ATP content observed in S.NNMT.LP (Figure 6) was not cytotoxic and that NNMT-V5 expression protected against NH-induced cell death. It is possible that the deleterious effects of NH on cell viability were not manifest over the time period of the experiment, hence prolonged incubation with NH may result in decreased cell viability and increased cell death being observed. In mitigation of this, the EC50_{MTT} for NH over the 120 h of the experiment (Figure 4) did not decrease; as such it is unlikely that further prolonged incubation would result in an increase in toxicity. It is possible that, although cell viability was not compromised, the reduction in cellular ATP in response to NH may leave the cell susceptible to further cytotoxic challenge, resulting in an eventual reduction in cell death.

MeNH was less toxic than NH towards both SH-SY5Y and S.NNMT.LP cells (Figures 4 & 6). This contrasts with findings from other studies which suggest that MeNH is more toxic than NH

[20,21,23]. There are a number of potential explanations for this observation. The first is that MeNH, as a charged molecule, was unable to enter the cell to elicit toxicity. This is supported by studies using HEK293 cells overexpressing DAT, which reported that the toxicity of MeNH relied upon DAT expression [23]. In contrast, NH would enter the cell *via* passive diffusion due to its relatively high lipophilicity ($\log P = 3.17$). Renal stem cell-derived HEK293 cells do not endogenously express DAT [49] and may thus demonstrate differing susceptibilities to BCs and their methylated forms compared to neuronally-derived cells. Furthermore, the DAT-containing plasmid used in Wernicke's study was a CMV promoter-based plasmid which, in HEK293 cells, would result in robust levels of protein expression [50], potentially resulting in supraphysiological levels of protein expression and so exaggerate the toxicity of MeNH. Storch and colleagues [51], using the same HEK293 model, demonstrated that there was no correlation between the V_{\max} of uptake kinetics of BCs and their cytotoxicity, nor was there a correlation between cytotoxicity and lipophilicity. In support of our observations, SH-SY5Y cells in their undifferentiated state express DAT [52,53] and as such are able to transport MeNH into the cell. Due to its positively charged nature, MeNH would sequester into the mitochondrion, raising the local concentration significantly [54]. Finally, many of the *in vivo* studies of BC toxicity use supraphysiological, acutely-administered concentrations of BC which do not mirror the gradual production and sequestering of MeNH modelled in our study.

The NNMT-V5-mediated protection against MeNH-induced reduction in cell viability and cellular ATP concentration in S.NNMT.LP cells after 120 h incubation (Figures 4 & 6) is in accord with our previous studies which have demonstrated similar protection against other CxI inhibitors [8,10]. More significant was the dose-dependent increase in cell viability and cellular ATP concentration in S.NNMT.LP by low concentrations of MeNH, an effect which was only manifest after 72 h incubation and which was not observed with either THNH or NH (Figures 6 & 7). This delayed increase in mitochondrial function may be an adaptive response of the cell to sub-lethal cytotoxic challenge. The mechanisms underlying this cytoprotection are as yet unknown, although the activation of the neuroprotective Akt signalling pathway in S.NNMT.LP [9] is an example of one such possibility. What is of note is that NNMT-V5 expression in S.NNMT.LP did not significantly increase MeNH's LC₅₀ compared to that of NH, which is in contrast to both the EC₅₀_{MTT} and EC₅₀_{ATP} and was not observed in SH-SY5Y cells (Figure 5). The LC₅₀ for MeNH was also significantly higher in SH-SY5Y compared to S.NNMT.LP. The reasons for these differences are unclear, however it does suggest that the toxicity of MeNH may not solely be mediated *via* CxI inhibition but may involve as-yet unidentified mechanism(s) which are specific to NNMT-expressing cells.

NNMT may be a detoxification pathway for β -carboline

This use of SH-SY5Y cells in PD-relevant neurotoxicity studies is controversial. Although widely used for studies such as ours [37], SH-SY5Y are pan-neuronal in their phenotype [9] and are tumorigenic in nature [55,56]. Nevertheless, undifferentiated SH-SY5Y cell do demonstrate some neuronal characteristics such as the expression of the presynaptic marker synaptophysin, the accumulation and release of dopamine and the expression of DAT [9,52,53]. Consequently, many studies use retinoic acid-based protocols to produce terminally-differentiated dopaminergic neurones [55-57], in which a number of proteins and signalling pathways are induced, for example Akt, which may affect toxicity. In both SH-SY5Y cell and PD animal models Akt induction has been shown to confer neuroprotection against the toxicity of the classic PD

neurotoxin 6-hydroxydopamine [52,58]. It is also unknown as to what effect differentiation has upon the expression of *N*-methyltransferases capable of *N*-methylating BCs. Thus, differentiation may mask the effects of NNMT-V5 upon BC toxicity that we wish to observe. The benefit of using the combination of SH-SY5Y and S.NNMT.LP is that they provide us with a true knock-in/knock-out model of NNMT expression and is ideal for the investigation of how NNMT influences the toxicity of BCs. Finally, the expression of NNMT-V5 in N27 rat mesencephalic neurones, an immortalised dopaminergic phenotype cell line, reproduced the effects of NNMT-V5 expression in SH-SY5Y [9], suggesting that many of the effects of NNMT-V5 expression in SH-SY5Y are likely to be replicated in neurones.

The observations that NNMT.V5-expressing cells are protected from NH-induced cell death, MeNH-induced reduction in cell viability and cellular ATP content, and that MeNH is less toxic than NH towards both cell lines suggests that NNMT may serve as a detoxification mechanism for BCs. There are caveats to this. Firstly, NH is a much poorer substrate than nicotinamide. Second, MeNH can be further converted to the more toxic species DMeNH. The enzyme responsible for this is unlikely to be NNMT for two reasons: (1) the lack of increase in toxicity observed for MeNH in S.NNMT.LP cells compared to SH-SY5Y, and (2) the lack of reports in the literature demonstrating that NNMT can *N*-methylate aliphatic nitrogen atoms. Therefore, confirmation of NNMT's detoxification of NH using pathophysiologically-relevant *in vivo* models will strengthen the evidence for a detoxification role for NNMT in the brain and confirm NNMT's central role in the regulation of cellular function and survival.

DECLARATION OF INTEREST

The authors declare that they have no conflict of interest with the contents of this manuscript.

AUTHOR CONTRIBUTION STATEMENT

MGT conducted the majority of the experiments and analysed the data. DS and ME produced the recombinant hNNMT^{WT} protein and wrote the paper with RBP. MvH assisted in the enzyme kinetic experiments. NIM wrote the paper with RBP. DMM assisted in the synthesis of NH and the interpretation of NMR data. DJB assisted in the analysis of the enzyme kinetic data. FK wrote the paper with RBP. DBR wrote the paper with RBP. MR conducted some of the experiments. RBP conceived the study, analysed the data with MGT and DJB, and wrote the majority of the paper.

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Tables.

	Toxin					
	THNH		NH		MeNH	
	SH-SY5Y	S.NNMT.LP	SH-SY5Y	S.NNMT.LP	SH-SY5Y	S.NNMT.LP
EC50 ₂₄	548 ¹ (523 ± 42) ²	521 (528 ± 23)	221 (221 ± 16)	199 (188 ± 14)	1370 (1389 ± 84)	759 (770 ± 8)
EC50 ₇₂	454 (474 ± 10)	327 (331 ± 6)	223 (228 ± 25)	288 (294 ± 18)	869 (881 ± 20)	619 (650 ± 36)
EC50 ₁₂₀	429 (432 ± 21)	366 (374 ± 31)	158 (162 ± 18)	155 (159 ± 18)	257 (237 ± 28)	355 (322 ± 21)
LC50	1880 (2068 ± 38)	2430 (2352 ± 52)	269 (270 ± 18)	406 (404 ± 10)	1830 (1820 ± 86)	355 (362 ± 48)
EC50 _{ATP}	437 (452 ± 80)	348 (335 ± 37)	263 (291 ± 41)	174 (225 ± 52)	260 (247 ± 56)	356 (383 ± 29)

Table 1. Summary of EC50 and LC50 values for 1,2,3,4-tetrahydronorharman (THNH), norharman (NH) and 2-N-methylnorharman (MeNH). ¹Values are the antilog of logEC50 or logLC50 values shown in Supplementary Table S4 and are expressed as μM . ²Values in brackets are the average of EC50 or LC50 values obtained from each experimental replicate and are expressed as $\mu\text{M} \pm \text{SEM}$. All values are the average of 3 – 5 experiments. Statistical analysis was performed using the logEC50 and logLC50 \pm SEM values shown in Supplementary Table S4.

THNH		Concentration (μM)							
		12.5	25	50	100	200	400	800	1600
MTT 24 h	SH-SY5Y	-	-	-	-	-	57 ± 6	16 ± 1	7 ± 2
	S.NNMT.LP	-	-	-	-	89 ± 3 [*]	85 ± 7	8 ± 0.7	4 ± 2
MTT 72 h	SH-SY5Y	-	-	-	-	-	47 ± 3	32 ± 3	19 ± 1
	S.NNMT.LP	-	-	-	-	-	30 ± 3	4 ± 2	1 ± 1
MTT 120 h	SH-SY5Y	-	-	-	-	-	66 ± 15 [*]	-0.4 ± 0.2	-0.9 ± 0.1
	S.NNMT.LP	-	-	-	-	-	42 ± 13	-0.7 ± 0.3	-0.7 ± 0.4
LDH	SH-SY5Y	-	-	-	-	-	-	-	58 ± 10
	S.NNMT.LP	-	-	-	-	-	-	-	34 ± 2
ATP	SH-SY5Y	-	-	-	-	-	33 ± 13	4 ± 3	0.4 ± 0.4
	S.NNMT.LP	-	-	-	-	-	25 ± 10	2 ± 0.8	0.6 ± 0.8

NH		Concentration (μM)							
		6.3	12.5	25	50	100	200	400	800
MTT 24 h	SH-SY5Y	-	-	-	-	82 ± 3 ^{**}	60 ± 2	13 ± 3	0.1 ± 0.2
	S.NNMT.LP	-	-	-	-	87 ± 5	51 ± 7	8 ± 2	0.3 ± 0.1
MTT 72 h	SH-SY5Y	-	-	-	-	73 ± 4	60 ± 7	10 ± 3	1 ± 0.2
	S.NNMT.LP	-	-	-	-	-	76 ± 7 ^{**}	24 ± 3	7 ± 0.7
MTT 120 h	SH-SY5Y	-	-	-	-	79 ± 8 [*]	35 ± 13	-0.8 ± 0.1	-0.6 ± 0.3
	S.NNMT.LP	-	-	-	-	-	30 ± 10	-0.7 ± 0.2	-1 ± 0.2
LDH	SH-SY5Y	-	-	-	-	-	39 ± 2	85 ± 6	94 ± 3
	S.NNMT.LP	-	-	-	-	-	33 ± 2	54 ± 0.6	100 ± 0.4
ATP	SH-SY5Y	-	-	-	-	-	-	2 ± 1	-0.1 ± 0.2
	S.NNMT.LP	-	-	-	-	-	39 ± 5	0.5 ± 0.5	-0.4 ± 0.4

MeNH		Concentration (μM)							
		15.6	31.3	62.5	125	250	500	1000	2000
MTT 24 h	SH-SY5Y	-	-	-	-	-	79 ± 2 ^{**}	65 ± 0.8	20 ± 8
	S.NNMT.LP	-	-	-	-	-	52 ± 2	18 ± 4	4 ± 3
MTT 72 h	SH-SY5Y	-	-	-	-	-	-	55 ± 3	0.2 ± 1
	S.NNMT.LP	-	130 ± 10	120 ± 7 [*]	-	-	-	6 ± 0.2	0.7 ± 0.4

MTT 120 h	SH-SY5Y	-	-	-	-	55 ± 2	11 ± 3	0.1 ± 0.2	-0.04 ± 0.1
	S.NNMT.LP	-	-	143 ± 4	164 ± 7	-	22 ± 8	0 ± 0.1	-0.04 ± 0.1
LDH	SH-SY5Y	-	-	-	-	-	-	-	73 ± 2
	S.NNMT.LP	-	-	-	-	-	99 ± 3	84 ± 4	89 ± 5
ATP	SH-SY5Y	-	-	-	-	46 ± 16	7 ± 4	0 ± 0.01	-0.4 ± 0.4
	S.NNMT.LP	-	-	-	140 ± 14 [*]	-	17 ± 4	0 ± 0.01	0 ± 0.01

Table 2 Summary of significant changes in cell viability (MTT), cell death (LDH) and intracellular ATP concentrations (ATP) in SH-SY5Y and S.NNMT.LP cells. For MTT and ATP experiments, results are expressed as percentage of untreated cells ± SEM. For LDH experiments, results are expressed as percentage cell death ± SEM. Only statistically significant results are shown and all are $P < 0.001$ unless otherwise indicated: * = $P < 0.05$, ** = $P < 0.01$.

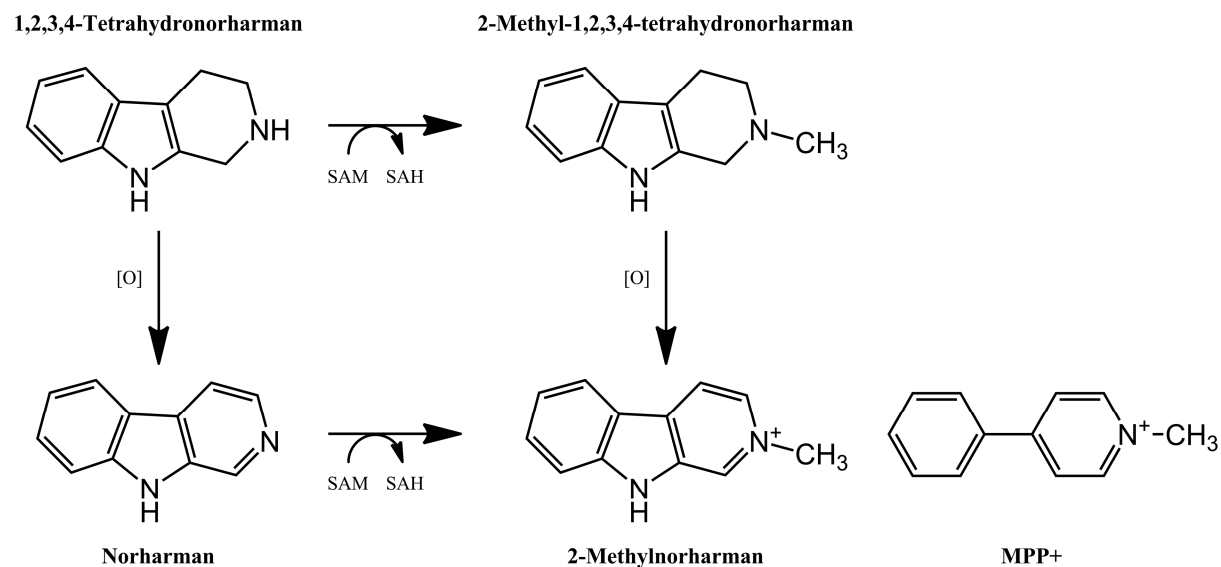


Figure 1. Metabolic pathways of the β -carboline norharman and the structural similarity of 2-methylnorharman to MPP+. Both compounds possess the same basic aromatic benzene and pyridine rings structure, with the addition of a bridging secondary amine between the two rings of 2-methylnorharman. SAM: *S*-adenosylmethionine, SAH: *S*-adenosylhomocysteine, [O]: oxidation.

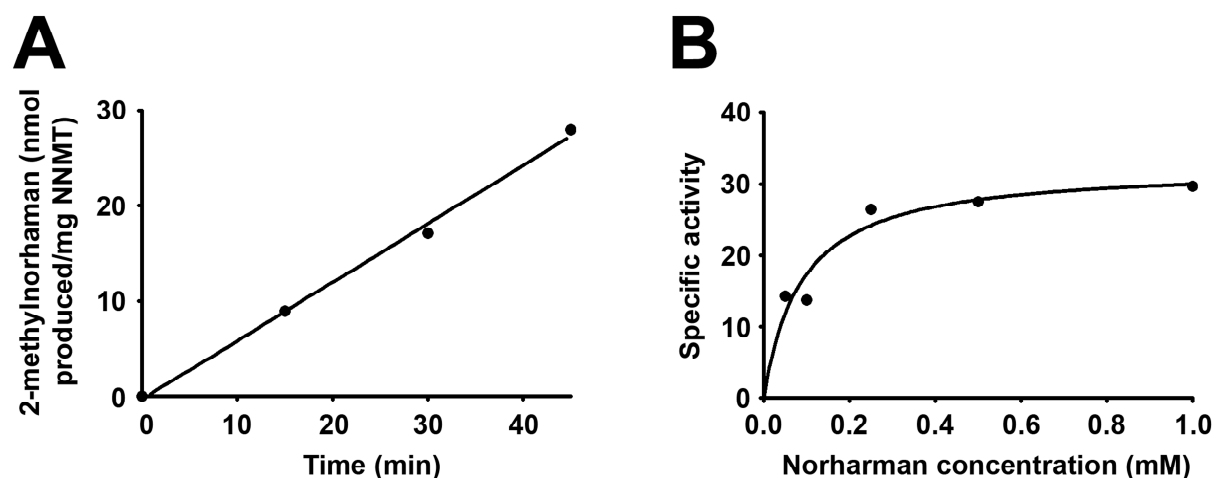


Figure 2. Biochemical analysis of norharman 2*N*-methyltransferase activity using a modified NNMT enzyme activity assay. Norharman was used as substrate, with detection of 2-*N*-methylnorharman performed using mass spectrometry. **(A)** Linearity of 2-*N*-methylnorharman production with time. **(B)** Non-linear regression analysis of norharman 2*N*-methyltransferase activity using Michaelis-Menten kinetic analysis. Specific activity is expressed as nmoles 2-*N*-methylnorharman produced/hour/mg hNNMT^{WT} protein.

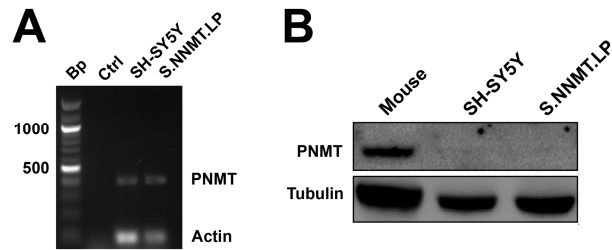


Figure 3. PNMT expression in SH-SY5Y and S.NNMT.LP cells. (A) Analysis of PNMT mRNA expression in SH-SY5Y and S.NNMT.LP cells using RT-PCR. (B) Analysis of PNNMT protein expression in whole mouse brain homogenate, SH-SY5Y and S.NNMT.LP cells using Western blotting. Bp: 100 bp ladder, Ctrl: no template control.

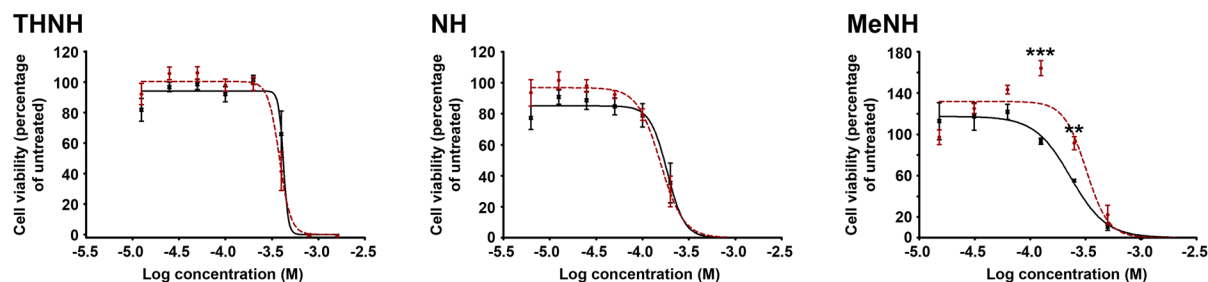


Figure 4. Toxicity of 1,2,3,4-tetrahydronorharman (THNH), norharman (NH) and 2-*N*-methylnorharman (MeNH) towards SH-SY5Y and S.NNMT.LP cells using the MTT reduction assay. Cell viability was expressed as percentage of untreated cells \pm SEM ($n = 5$ for THNH and NH, $n = 4$ for MeNH). Curve fitting was undertaken using non-linear regression analysis. Statistical analysis of individual dose toxicity between cell lines consisted of two-way repeated measures ANOVA: ** = $P < 0.01$, *** = $P < 0.001$. Solid black line = SH-SY5Y, dashed red line = S.NNMT.LP.

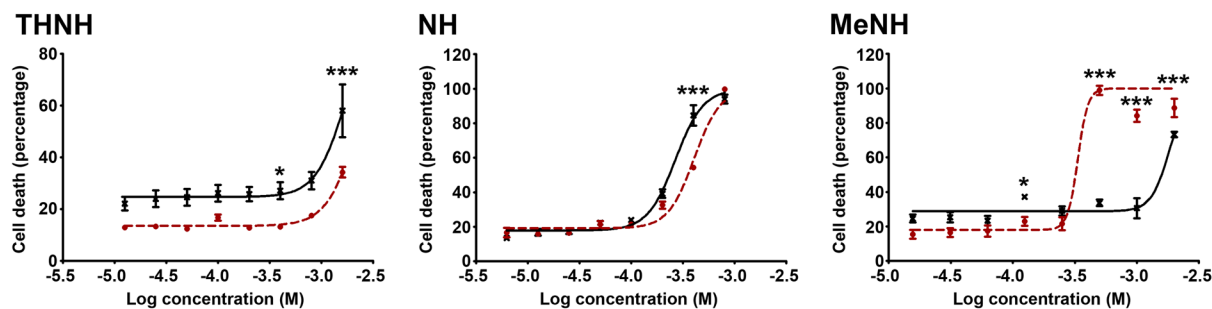


Figure 5. Effect of 1,2,3,4-tetrahydronorharman (THNH), norharman (NH) and 2-N-methylnorharman (MeNH) upon cell death using the LDH cytotoxicity assay. Cell death was expressed as percentage cell death \pm SEM ($n = 3$ for all). Curve fitting was undertaken using non-linear regression analysis. Statistical analysis of individual dose toxicity between cell lines consisted of two-way repeated measures ANOVA: * = $P < 0.05$, *** = $P < 0.001$. Solid black line = SH-SY5Y, dashed red line = S.NNMT.LP.

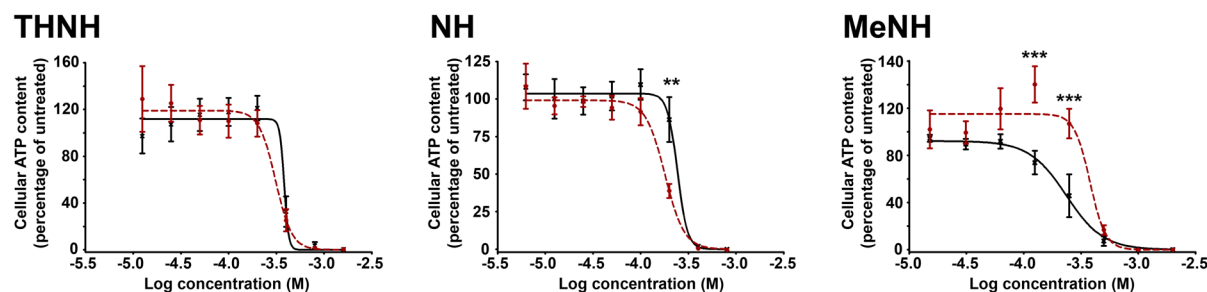


Figure 6. Effect of 1,2,3,4-tetrahydronorharman (THNH), norharman (NH) and 2-*N*-methylnorharman (MeNH) upon cellular ATP content in SH-SY5Y and S.NNMT.LP cells. Cellular ATP content was expressed as percentage of untreated cells \pm SEM ($n = 5$ for THNH and NH, $n = 4$ for MeNH). Curve fitting was undertaken using non-linear regression analysis. Statistical analysis of individual dose toxicity between cell lines consisted of two-way repeated measures ANOVA: ** = $P < 0.01$, *** = $P < 0.001$. Solid black line = SH-SY5Y, dashed red line = S.NNMT.LP. Cellular ATP content in untreated cells: SH-SY5Y = 150 ± 12 pmoles/mg protein, S.NNMT.LP = 310 ± 54 pmoles/mg protein.

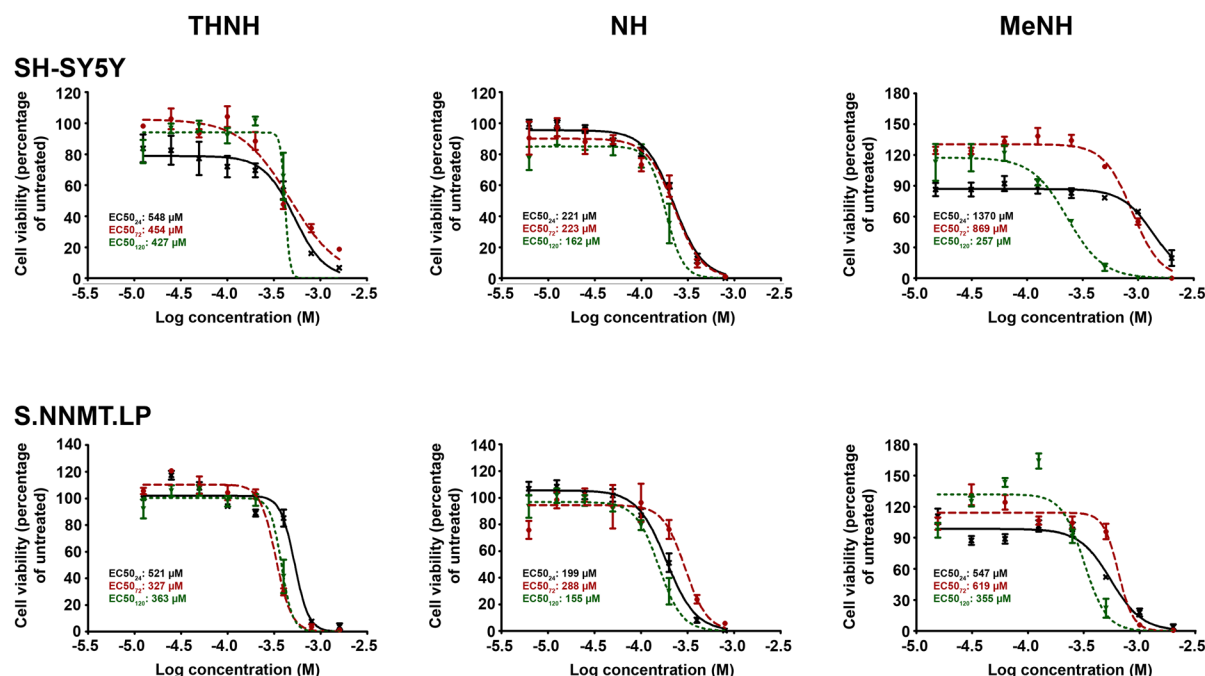


Figure 7. Effect of length of incubation with 1,2,3,4-tetrahydronorharman (THNH, left hand panels), norharman (NH, middle panels) and 2-*N*-methylnorharman (MeNH, right hand panels) upon cell viability measured using the MTT reduction assay, and comparisons of EC₅₀s, in SH-SY5Y (top three panels) and S.NNMT.LP (bottom three panels) cell lines. Cell viability was expressed as percentage of untreated cells \pm SEM ($n = 4$ for NH and MeNH, $n = 3$ for THNH). For all panels: solid black line = 24 h, large dashed red line = 72 h, small dashed green line = 120 h.